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Two improved promoter sequences for the β-lactamase expression arising from a single base-pair substitution

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ABSTRACT

A mutation in the transposon Tn2660 derived from the plasmid R6K, and resulting in an approximate tenfold increase in penicillin resistance is shown to be a single GC to AT substitution 177 base pairs 'upstream' of the initiation codon of the structural β-lactamase (bla) gene. This substitution leads to the transcription of two new mRNAs which can be ascribed to the creation of two new overlapping promoter sequences. All the sequences (450bp) examined in the wild-type Tn2660 are identical to those reported in Tn3.

INTRODUCTION

In a previous publication (1) we have reported the isolation of a mutant, pTY101, of the plasmid R6K (2). R6K mediates resistance to streptomycin and to penicillins and, under its earlier name R(TEM)(3), this latter resistance was shown to arise by the synthesis of a β lactamase (penicillin amido-β-lactamhydrolase, EC3.5.2.6), designated TEM, and characterized by isoelectric focussing as a TEM-1 type β lactamase (4). The ampicillin resistance of R6K was later determined to reside on a transposon which has extensive homology with Tn3 (5), and this transposon, designated Tn2660, has been the subject of a number of genetic studies (1,6-10). The sequence of the structural gene of β lactamase on Tn3 and 210 base pairs upstream of the initiating codon are incorporated as part of the structure of the cloning vector pBR322 (11,12). Several reports have recently appeared on the identification of promoters for β lactamase carried on pBR322 (13,14,15).

pTY101 was derived from R6K by selection for hyperresistance to ampicillin, resulting in the acquisition of a second copy of Tn2660, probably by a mechanism of intramolecular transposition. However, in contrast to most events of this kind which led to an approximate doubling in the ampicillin resistance level, pTY101 was found to have an approximate ten-fold increase (1). Preliminary experiments (8,10 and unpublished) in which the two transposons of pTY101 were cloned into separate ColE1

replicons, showed that one transposon produced the normal resistance level, whereas the other, which was designated Tn2661, resulted in a greatly elevated level.

This publication reports the further analysis of the transposon producing increased resistance. It is shown by the Berk and Sharp (16) method that two new messenger RNAs initiated well upstream of the β-lactamase structural gene are transcribed in strains carrying Tn2661. Base-sequence determinations of the DNAs from Tn2660 and Tn2661 in the region of initiation of these messengers show the sequence of Tn2660 to be identical to that of Tn3 (11,12), but there is a single base-pair substitution in Tn2661, which can account for its increased resistance. Other base-sequence determinations of Tn2660 in the amino-terminal coding region for β lactamase also show identity with Tn3.

MATERIALS AND METHODS

Bacterial strains and plasmids. The R6K plasmid (2) and its pTY101 mutant (1) have been previously described. pBR322 is a laboratory construct (17). pA03 is a small (1683bp), fully-sequenced, truncated derivative of ColE1 (18). Both pBR322 and pA03 appear to have similar copy numbers (17,18). Host strains were the recA derivative, RC655, of the Lac$^-$ E. coli K12 strain, ED2117 (19), and CGSC5924, a thyA metE deoC polA strain (8).

Genetic techniques, isolation of plasmid DNA and agarose-gel electrophoresis have been previously described (1,8,9). Restriction enzymes were purchased from Bethesda Research Laboratories.

RNA preparations were derived essentially by the method of Dennis and Nomura (20). This method results in the isolation of the total cellular RNA. However, due to the specificity of the hybridization step, only messenger RNA transcribed from the DNA sequence under investigation will hybridize and protect it from degradation by S1 nuclease. Preparations of total RNA were derived from cultures of the host strains RC655 carrying either pA03::Tn2660, or pA03::Tn2661, and will thus be referred to as Tn2660 mRNA and Tn2661 mRNA, respectively. As a control, a similar total RNA preparation was isolated from the same host strain which carried pBR322.

S1-nuclease mapping of RNA followed the Berk and Sharp (16) method. The initial DNA probe was the 634bp HpaII fragment, cleaved either from pA03::Tn2660 or pA03::Tn2661, dephosphorylated by calf-intestine alkaline phosphatase (21) (Sigma Chemical Co.), and separated on a 5% polyacrylamide gel. It was then purified by electroelution and column chromatography on

Whatman DE52, and labelled at the 5'-termini with T4-polynucleotide kinase (Bethesda Research Labs) and [γ-^{32}P]ATP (4500 ci/m mole, ICN) (22,23). A second DNA probe was derived from the 634bp HpaII fragment by cleavage with HhaI to produce four fragments of 13bp, 263bp, 332bp and 26bp (Fig. 2). Following dephosphorylation, the 263bp fragment was isolated, purified as described above and labelled at the 5' termini by similar procedures (23). This labelled fragment was then cleaved with HaeIII to produce fragments of 14bp, 36bp, 128bp and 85bp, and the terminal 85bp fragment, labelled at the 5' end at coordinate 3844, was similarly separated (see Fig. 2C).

Labelled DNA fragments (approximately 5 to 7ng, 15,000 cpm of the 634bp fragment, or 3 to 4ng, 4,000 cpm of the 85bp fragment) and total RNA (45 to 150 µg hybridized with the 634bp probe, or 75 to 100 µg hybridized with the 85bp probe) were mixed, precipitated in ethanol and after pelleting and drying in vacuum, were resuspended in 30 µl hybridization buffer [40 mM PIPES, 1mM EDTA, 400 mM NaCl, 80% (vol/vol) formamide at pH 6.4] and heated at 72°C for 15 min to denature the DNA, then held at 50°C for 3 hr using the 634bp probe, or at 40°C for 3 hr using the 85bp probe, to permit specific formation of RNA/DNA hybrids. 87 units of S1 nuclease (P-L Biochemicals) in 0.3 ml of ice-cold buffer (280mM NaCl, 50mM sodium acetate, 4.5mM $ZnSO_4$, pH 4.6) was added and the mixture held at 37°C for 30 min. The nucleic acids were then reprecipiated with ethanol, suspended in 10 µl gel-loading buffer (90% formamide, 10mM NaOH, 0.01% bromophenol blue, 0.01% xylene cyanol FF), and after heating at 100°C for 2 min to denature double-stranded nucleic acids, were loaded on a 7M urea, 5% (634bp probe) or 8% (85bp probe) polyacrylamide gel. After electrophoresis, the location of the S1-nuclease-resistant, labelled DNA probe was detected by autoradiography.

Base-sequencing of DNA was carried out on DdeI fragments of the 634bp HpaII fragment and also on the 85bp HaeIII fragment by the standard method of Maxam and Gilbert (23).

RESULTS

Separation of transposons of pTY101 and identification of Tn2661

We have previously reported (8) the cleavage of pTY101 by SmaI and EcoRI into three fragments, a 14.6kb fragment carrying the R6K replication genes, a 21.0kb fragment carrying one ampicillin resistance (Ap-r) transposon and the streptomycin-resistance (Sm-r) gene(s) of R6K, and a 7.9kb fragment carrying the other Ap-r transposon. The 21.0kb fragment was cloned in a ColE1 replicon to produce pSJC116 (8). The 7.9kb fragment was

also cloned in a ColE1 replicon to produce the plasmid pSJC104 (see Fig. 1). The ampicillin resistances of pSJC116 and pSJC104 in the recA host RC655, expressed as LD37 values, were found to be 2.8mg/ml and 0.25mg/ml, respectively. We concluded that pSJC116 carries a mutant transposon with increased β-lactamase resistance which we designate Tn2661.

Since pSJC116 and pA03 are both ColE1 replicons, and therefore incompatible, in order to achieve transposition of Tn2661 into pA03, it was first transposed from pSJC116 into pSJC101 [a R6K replicon carrying the kanamycin resistance (Km-r) region of pML2 (1,24)], by transforming a RC655(pSJC116) strain with pSCJ101 plasmid DNA, and selecting transformants which were Ap-r, Sm-r and Km-r (Fig. 1). The plasmid DNA from one transformant, when isolated and characterized on an agarose gel, was found to comprise two DNA molecules the sizes of pSJC116 and pSJC101. The plasmid DNA from a culture of this RC655(pSJC116,pSJC101) transformant was then used to transform

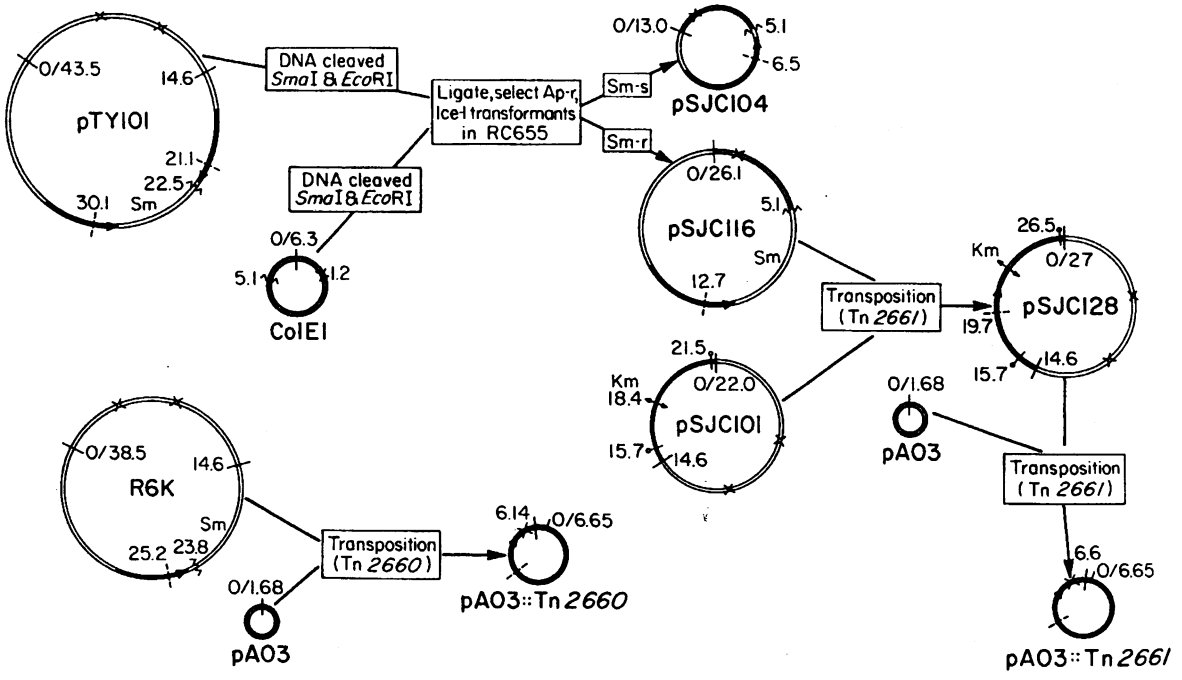

Figure 1: Derivation of derivative plasmids of pA03 carrying the wild-type transposon Tn2660 and the mutant transposon Tn2661.

Double-line circles represent plasmid DNA, with the transposons shown shaded and arrowheads at the IR(R) terminus (12). R6K sequences are otherwise non-shaded, and the ColE1, pML2 and pA03 sequences are shown stippled. Figures are coordinates in kilobase (kb) pairs numbered clockwise. Intercepts (|), (⁞), (⧘), (⇕), and (⚲) show recognition sites for the restriction endonucleases EcoRI, BamHI, SmaI, HindIII and KpnI respectively. Sm, Km, Ap represent resistance to streptomycin, kanamycin and ampicillin respectively, Icel represents immunity to colicin El, and the ⋈ symbol represents replication origins of R6K or ColE1 (18,32,33).

competent CGSC5924(polA) cells, in which pSJC116, a ColE1 replicon, cannot grow (25), selecting for Km-r, Ap-r transformant clones. One Km-r, Ap-r transformant was selected, and the plasmid DNA was isolated and shown by BamHI cleavage and agarose-gel electrophoresis to be a single molecular species of 27kb, consistent with the transposition of Tn2661 into pSJC101 (Fig. 1). This was confirmed by further cleavage with EcoRI and BamHI and analysis of fragments on agarose gels, and the plasmid construct was designated pSJC128.

Derivation of small, sequenced plasmids carrying Tn2660 and Tn2661

Assuming that the muation in Tn2661 resulted from a change in transcription, we chose to compare the sites of promoters initiating transcription in Tn2660 and Tn2661. From the published data of Sutcliffe on pBR322 (11) and of Heffron et al. on Tn3 (12), it may be inferred that the segment of non-translated DNA on Tn3 immediately upstream from the initiation codon for the β-lactamase enzyme can be readily isolated on a fragment following cleavage by HpaII, since this is the largest HpaII cleavage fragment of Tn3. Since Tn2660 is known to resemble Tn3 in overall homology (5), transposition (8) and limited base-sequence determinations (10), and preliminary experiments have indicated similar restriction-enzyme cleavage sites on Tn3 and Tn2660, it was assumed that a corresponding HpaII fragment of Tn2660 would also carry a similar non-translated region. In order to be able to predict the sizes of all HpaII-cleavage fragments from a plasmid carrying Tn2660 or Tn2661, so that a unique HpaII-cleavage fragment with the amino-terminal coding region of the bla gene could be isolated, we transferred these transposons to a small, sequenced plasmid, pA03 (18).

Final transposition of Tn2661 into pA03 was achieved in two steps. Firstly, a strain carrying the two plasmids pSJC128 and pA03 was constructed, by using pSJC128 DNA to transform an E. coli recA host strain (RC655) into which pA03 had previously been transformed, and selecting for Ap-r and Km-r (carried on pSJC128), and immunity to colicin E1(Icel), a property of pA03. DNA was then isolated from this biplasmid host and added to competent RC655 host cells, selecting transformants for Ap-r and Icel, and then screening by replica plating for Km-s transformants (since Ap-r, Icel, Km-s transformants have the phenotype expected of a pA03 plasmid into which Tn2661 had transposed: Fig. 1). This was confirmed by the isolation of plasmid DNA from a number of transformants and determining their size to be 6.7kb, the sum of the sizes of pA03 (1.7kb) and Tn2660 (5kb). This was achieved by linearizing the plasmid DNAs by cleavage with BamHI, and

comparing their mobilities on an agarose gel against fragments of known size derived by EcoRI cleavage of DNA. One such transformant harboring a plasmid of 6.7kb was selected and the plasmid was designated pA03::Tn2661. Similarly, pA03::Tn2660 was isolated by a similar procedure using a host strain carrying R6K as a source of Tn2660 (Fig. 1).

Both pA03::Tn2661 and pA03::Tn2660 were characterized by cleavage with EcoRI and BamHI, or HpaII. The sizes of HpaII fragments that may be inferred from the base sequence of Tn3 (12) (and hence from Tn2660) should be 634bp, 408bp, 304bp (IRR terminus), together with 33 smaller fragments (including the IRL terminal fragment of 199bp). Those of pA03 are 768bp, 205bp and 7 smaller fragments (18). Following agarose-gel analysis of the HpaII-cleavage fragments from the DNA of pA03::Tn2660 or pA03::Tn2661, it was clear that in both cases, the fragment sizes, at least those of the larger fragments, conformed to those expected, and were consistent with transposition into the major 768bp HpaII fragment of pA03. Base-sequence analysis of DdeI-cleavage fragments of the junction regions showed Tn2660 is inserted with a duplication of the 1102 to 1106 pA03 sequence, and Tn2661 is inserted with duplication of the 1578 to 1582 sequence of pA03. The ampicillin resistances conferred on the same RC655 host strain by pA03::Tn2660, pA03::Tn2661 or pBR322 were measured as LD37 values of 0.2, 2.2 and 1.2 mg/ml respectively, confirming the previous measurements on pSJC104 and pSJC116. The relative copy numbers of pA03, pA03::Tn2660, pA03::Tn2661 and pBR322 measured by assay of plasmid DNA by agarose-gel electrophoresis (26) showed no major differences.

Fig. 2A shows the structure of pA03::Tn2660. The HpaII junction fragments were identified as 481bp and 795bp, whereas in pA03::Tn2661, they are 319bp and 957bp. In both cases, the 634bp fragment can be readily identified as a well-separated, second-largest fragment. This fragment was isolated from pA03::Tn2661 and pA03::Tn2660 and, after ^{32}P end-labelling, these fragments were used as DNA probes in protection studies by messenger-RNA. The 634bp fragments from pA03::Tn2660 and pA03::Tn2661 were further cleaved and labelled to produce the 85bp probe (Fig. 3c), labelled only at the 3844 terminus in the 5' end.

mRNA protection of DNA probes (Berk & Sharp)

The ^{32}P-labelled DNA probes were denatured, mixed and hybridized at 50°C (634bp probe) or 40°C (85bp probe) with RNA preparations derived from strains carrying either pA03::Tn2660, pA03::Tn2661 or pBR322, including RNA from pA03::Tn2661 in two different amounts. The DNA/RNA hybrid preparations

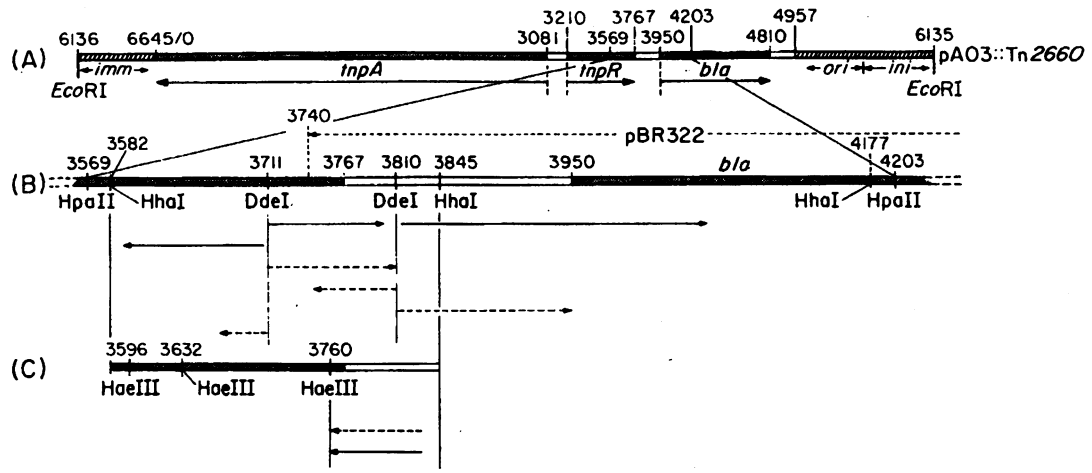

Figure 2: (A) The double line represents the DNA of plasmid pA03::Tn2660 shown as a linear molecule cleaved at the EcoRI site of pA03 (18). The open and shaded segments are Tn2660 DNA and the cross-hatched segments are pA03 DNA. The numbers above the line represent base-pair coordinates derived from the base sequences of Tn3 (12) and pA03 (18), with the junction between the inverse repeat (left) IR(L) terminus of Tn2660 and pA03 designated as the zero coordinate. (The numbers of Tn2660 DNA thus correspond to those designated on Tn3.) tnpA, tnpR, and bla represent the transposase, resolvase/repressor and β-lactamase genes of Tn3 and Tn2660, and the arrows show the regions and directions of transcription of these genes. ini, ori and imm represent the replication, initiation and origin regions of pA03 and the colicin E1 immunity region respectively.

A similar figure can be drawn for pA03::Tn2661 with the difference that the EcoRI site is located at coordinate 6612 referred to the junction point of transposition of the IR(L) terminus of Tn2661 into pA03 as 0/6645.

(B) The unique 634bp HpaII fragment of pA03::Tn2660 or pA03::Tn2661. The broken line with the arrow and pBR322 represents the segment of Tn3 that is incorporated in pBR322 (11). The sites of cleavage by DdeI are shown at coordinates 3711 and 3810, and by HhaI at coordinates 3582, 3845 and 4177, and the horizontal lines with arrows below indicate the regions for which we determined the DNA sequence; unbroken lines show Tn2660 sequences and broken lines Tn2661 sequences.

(C) The 263bp 5'-labelled HhaI fragment, which was isolated and recleaved with HaeIII at coordinates 3596, 3632 and 3760. The 85bp fragment (coordinates 3760 to 3845), labelled at the 5' end of its 3844 terminus was used as a second probe, and was also sequenced as indicated.

were treated with S1 nuclease to digest the non-hybridized nucleic acid segments and, following denaturation, the single-strand truncated DNAs were run on 7M urea polyacrylamide gels and their sizes determined by autoradiography. As controls, the DNA probes were run following, (a) the initial denaturation step, (b) denaturation and S1-nuclease digestions without hybridization with mRNA, and (c) denaturation and S1-nuclease digestion after incubation under hybridization conditions without mRNA.

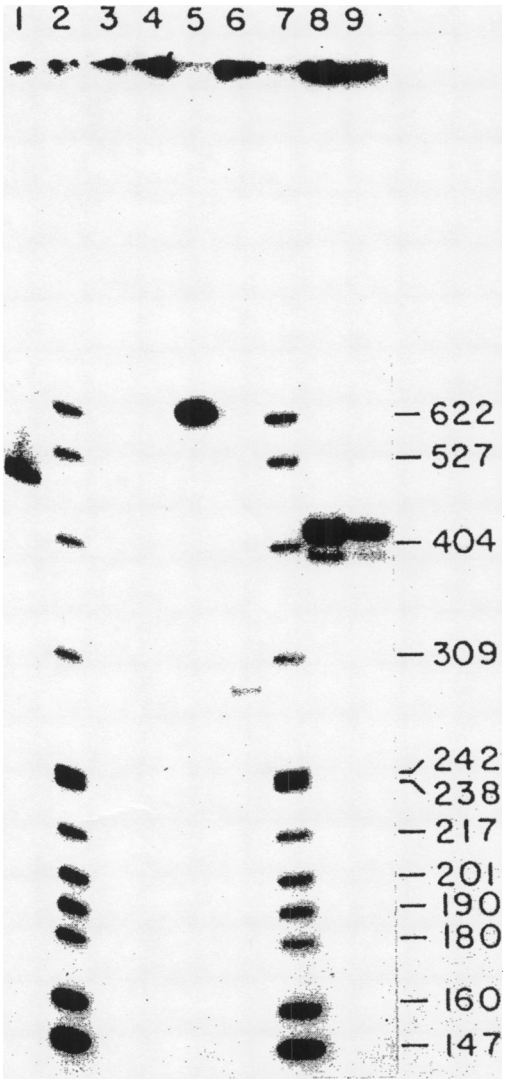

Figure 3: Initiation sites for bla mRNA determined by protection of ^{32}P-labeled 634bp fragment of pA03::Tn2660 following hybridization with mRNAs from pA03::Tn2660, pA03::Tn2661 or pBR322 and S1-nuclease treatment. A 7M urea 5% polyacrylamide gel in TBE buffer was run under constant power (40W) for 2 hr. Following electrophoresis, the gel was exposed to Kodak XAR-2 film at -20° overnight.

Lanes 2 and 7 contain pBR322 DNA cleaved with HpaII, and labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP as a size standard (sizes of fragments in nucleotides are shown at side). Other lanes contained the DNA probe hybridized with mRNA and treated by Berk and Sharp method (16) as follows:

Lane #	DNA (cpm)	RNA Source	RNA Amount (µg)
1	15,000	pBR322	75
3	15,000 [1]	none	0
4	15,000 [2]	none	0
5	5,000 [3]	none	0
6	15,000	pA03::Tn2660	150
8	15,000	pA03::Tn2661	90
9	15,000	pA03::Tn2661	45

[1] denatured probe, S1 digestion following incubation without mRNA.

[2] denatured probe, S1 digestion without hybridization

[3] denatured probe

The results of hybridization of the 634bp DNA probe from pA03::Tn2660 with mRNAs from Tn2661, Tn2660 and pBR322 are shown in Fig. 3. It may be seen that hybridization with mRNA from Tn2660 results in a band of approximately 285 nucleotides (with faint bands of approximately 395 and 420 nucleotides). Hybridization with mRNA from Tn2661 produced an intense band of 420 and a strong band of 395 nucleotides and there was a proportionate decrease in the intensity of both bands when only half the amount of RNA was used. In other experiments (not shown) in which greater amounts of RNA from Tn2661 were hybridized, a faint band of approximately 285 nucleotides was seen. Results using the DNA probe from pA03::Tn2661 with mRNAs from Tn2660, Tn2661 and pBR322 (data not shown) gave essentially similar autoradiographic data in all respects.

It may thus be concluded that there are two new, or greatly amplified mRNA transcripts from Tn2661, a major transcript hybridizing with approximately 420 nucleotides of either 634bp DNA probe, and a minor transcript hybridizating with about 395 nucleotides of either 634bp probe. In contrast, the predominant mRNA from Tn2660 protected a region of both DNA probes to a total of approximately 285 nucleotides. The 285 nucleotides protected in Tn2660 is equivalent to the distance from the HpaII terminus at coordinate 4203 to the 3918 coordinate. This corresponds closely to the site of initiation of β-lactamase mRNA transcription in the segment of the Tn3 transposon carried on pBR322 as identified by Russell and Bennett (13) at coordinate 3915, and confirmed by Brosius et al. (14), and which is consistent with the approximate position of the promoter on pBR322 designated as P3 by Stuber and Bujard (27). In the experiments using protection by Tn2661 mRNA, if we assume that transcription is from the β-lactamase coding strand, then the major band corresponds to initiation at approximately 3783bp, and the minor band to initiation at approximately 3808bp.

The intensities of the autoradiographic bands on Fig. 3 were measured by a Joyce-Loebl microdensitometer. The results showed that the relative intensities of the traces per unit weight of total RNA in the hybridization reactions were 0.15 (420 nucleotides), 0.2 (395 nucleotides) and 0.7 (285 nucleotides) for Tn2660; 6.8 (420), and 1.7 (395) for Tn2661 (90μg), 7.3 (420) and 1.7 (395) for Tn2661 (45μg), and 5.0 (460 nucleotides) for pBR322. The equivalence of band intensity per unit weight of RNA from experiments using either 45μg or 90μg of RNA from Tn2660 indicated that the amount of DNA used (5ng) was in excess and this was confirmed by essentially similar data using 7ng of the DNA probe from pA03::Tn2661.

The sum of the relative intensities of the bands from Tn2661 is 8.7-fold those from Tn2660, and that from pBR322 is 5-fold those in Tn2660. These values compare with the relative ampicillin resistances (LD37s)of host strains carrying these elements, since Tn2661 was found to produce an 11-fold and pBR322 a 6-fold greater resistance than Tn2660.

The results of hybridization of the 85bp probe from pA03::Tn2660 with mRNAs from Tn2661 and Tn2660 are shown in Fig. 4. As expected, these data show no protected bands and thus no initiation in the 85bp HaeIII fragment of mRNA from Tn2660, but in the case of mRNA from Tn2661, four major bands and four minor bands can be seen. From the base-sequence reactions of Tn2660 and Tn2661 DNA run on the same gel, the position of the four major

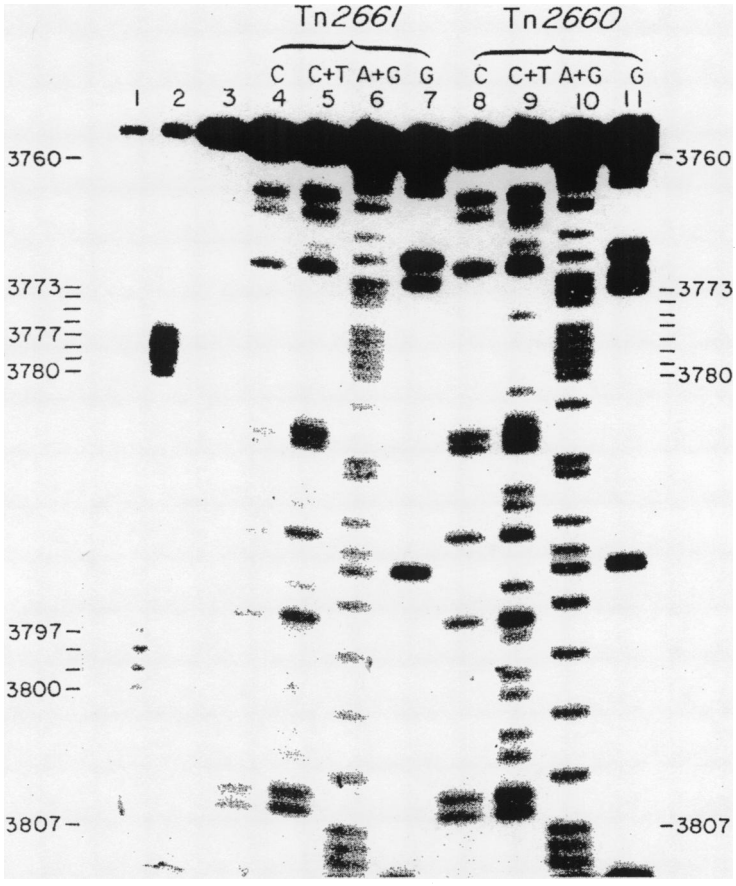

Figure 4: Base sequences and initiation sites for transcription on the 85bp HaeIII fragments from Tn2660 or Tn2661

A 7M urea 8% polyacrylamide gel in TBE buffer was run under constant power (50W) for 2 hr. Following electrophoresis, the gel was exposed to Kodak XAR-2 film at -70°C for 5 days. Lanes 1 and 2 were loaded with the 85bp DNA fragment from Tn2661 following hybridization with mRNA, either from pA03::Tn2660 (lane 1) or pA03::Tn2661 (lane 2) and subsequent S1-nuclease treatment. DNA sequencing reactions of Tn2661 DNA are shown in lanes 4 to 7, and of Tn2660 DNA in lanes 8 to 11. Lane 3 is a control denatured probe from Tn2661. In both hybridization experiments, 4000 cpm of DNA probe was used.

bands can be fixed at coordinates 3777 to 3780, and the minor bands at coordinates 3797 to 3800.

DNA base sequencing

Since the mRNA protection experiments indicated new or greatly enhanced initiation sites for mRNA transcription in Tn2661 approximately 145 to 170 nucleotides upstream of the initiation codon for the β-lactamase gene, we wished to determine whether a base-sequence change(s), consistent with the establishment of new promoter recognition sites had occurred in Tn2661. The HpaII fragment of Tn2661 and that of Tn2660 was isolated as described and

then subjected to cleavage by DdeI, resulting in three fragments of 142bp (3569 to 3711), 99bp (3711 to 3810) and 393bp (3810 to 4203). Segments of the coding and non-coding strands of each of these fragments were sequenced by the Maxam and Gilbert technique in the regions shown in Fig. 2.

The 85bp HaeIII nucleotide sequence of Tn2660 and Tn2661 was also determined and the results are also shown in Fig. 4. A comparison of these sequences and other determined sequences of the 634bp HpaII fragment show that between the 3585 and 4023 coordinates, the base sequence of Tn2660 corresponds to that published for Tn3 (12). In the case of Tn2661, this sequence was identical, except that at coordinate 3773, T was substituted for C on the non-coding strand, and A was substituted for G on the coding strand (see Figs. 4 and 5).

DISCUSSION

RNA-polymerase recognition sequences on Tn2660 and Tn2661

As might be expected from the close homology of Tn3 and Tn2660, their similarity in transposition function (1,8,9) and the homology of their base sequences in the terminal regions (10), it is not surprising that the base sequence in the non-translated region between tnpR and bla (and indeed over the whole region studied) should correspond in Tn2660 to that determined for Tn3 (see Fig. 5).

Tn2661 has the same sequence, but has a TA pair substituted for a CG pair at coordinate 3773, which is concluded to result in the initiation of two new messengers in high amounts from the approximate coordinates 3777 to 3780 and 3797 to 3800. Other authors (e.g. 14) have also shown that the sizes of probes protected by RNA transcripts from S1-nuclease digestion vary over three or four nucleotides. These same authors have also shown by in-vitro labelling that the transcript can be localized to one of these nucleotides, indicating that S1 digestion does not terminate precisely at the double-stranded region.

The base sequence of Tn2660 from coordinate 3768 to 3773 is TACGCC, and in Tn2661 it has mutated to TACGCT, the latter sequence now corresponding in three of its sites to the three most conserved bases of the canonical Pribnow box, TATAAT (28). Eighteen bases upstream from the first T of this box is the sequence TTGAA, which corresponds in the first four of its bases to the canonical "-35 sequence", TTGAC (29,30). A comparison of the -35 and -10 boxes for this proposed new promoter (Pa) of Tn2661, which is consistent with initiation of the major new RNA species at the approximate 3777 to 3780

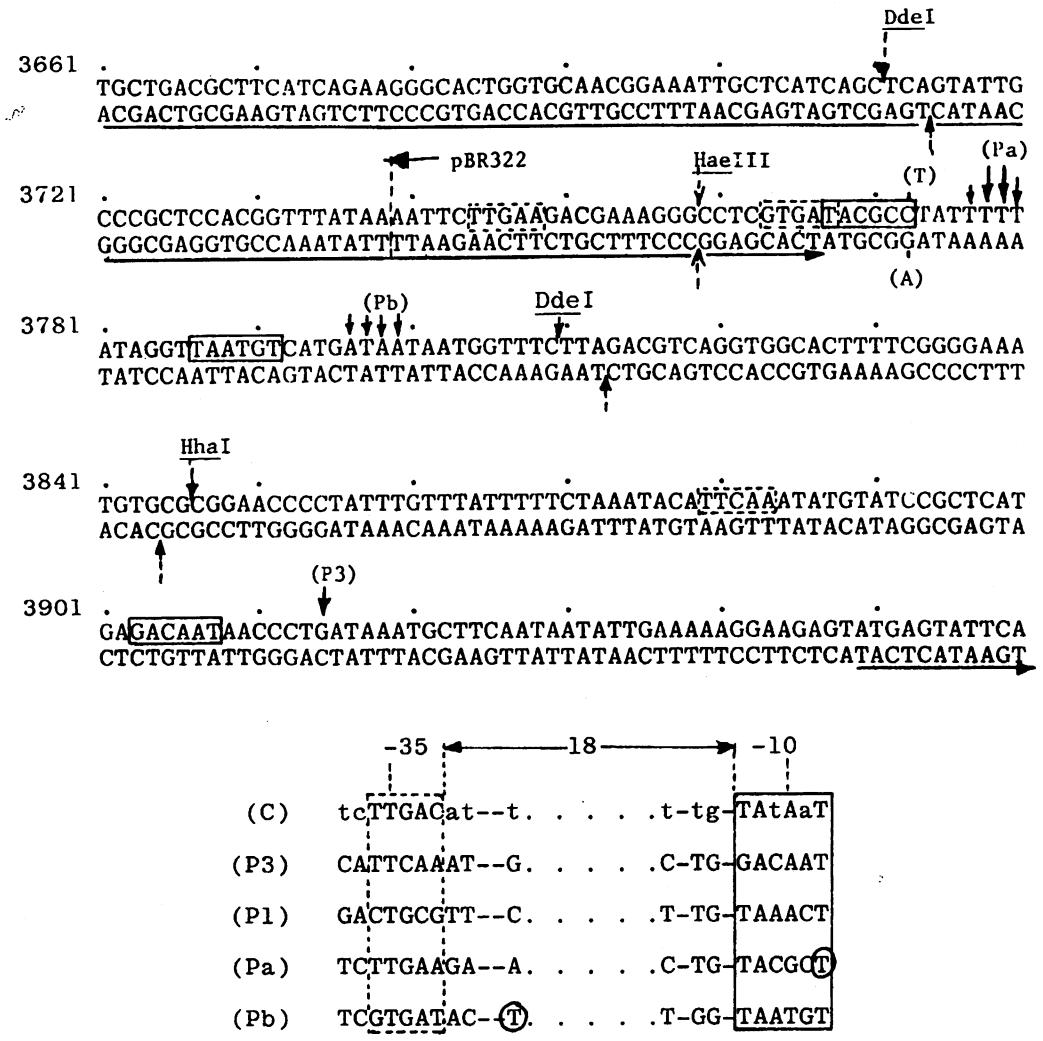

Figure 5: (A) Part of the base sequence of Tn2660 determined from coordinates 3661 to 3960. [The coordinate numbers and base sequence corresponds precisely to those determined for Tn3. Cleavage sites for DdeI, HaeIII and HhaI are shown by broken vertical lines and the sequence from coordinate 3740 is carried on pBR322. The arrows shown under sequences 3661 to 3767 and 3950 to 3960 indicate parts of the translated regions of Tn3 for tnpR and bla genes respectively (11,12).] The base sequence of Tn2661 was found to be identical except that at coordinate 3773, the CG pair was substituted by a TA pair. The boxed sequence, GACAAT (3903 to 3908) represents the 'Pribnow box' for the wild-type β-lactamase promoter of Tn2660, and the broken-boxed sequence, TTCAA (3880 to 3884) the -35 region for this same promoter, P3 (27), initiating transcription at coordinate 3915 (These sequences and coordinates are identical to those previously specified (13,14).) The boxed-sequence, TACGCT (3768 to 3773) and the broken-boxed sequence TTGAA (3745 to 3749) are identified as a new or greatly enhanced promoter (Pa) on Tn2661 giving rise to an approximate tenfold increased level of transcription compared to P3, initiating at the approximate coordinates 3777 to 3780. The boxed-sequence, TAATGT (3787 to 3792) and the broken-boxed sequence, GTGAT (3764 to 3768) are identified as a second new or enhanced promoter (Pb) on Tn2661 giving rise to an approximate twofold increased level of transcription compared to P3 and initiated from the

approximate coordinates 3797 to 3800. The unbroken vertical arrows indicate the approximate coordinates at which β-lactamase mRNA was initiated in Tn2660 (3915), and Tn2661 (3777 to 3780 and 3797 to 3800).

(B) The promoter regions of Tn2660 and Tn2661 compared with the P1 β-lactamase promoter for pBR322 (14) and the 'canonical' promoter sequences (30). (In all cases the -35 sequence has been chosen so that there are 18 base pairs between the -35 and -10 boxed sequences.) Pa, Pb represent the high-level and medium-level transcription promoters in Tn2661, P3, the natural promoter sequence of Tn3 (also present in Tn2660, Tn2661 and pBR322), P1, the second β-lactamase promoter in pBR322 (14) and C, the canonical promoter sequence (30) in which the more conserved nucleotides are designated by capital letters and the less conserved nucleotides are shown in lower case. The nucleotide circled is that arising by mutation to produce Tn2661 from Tn2660.

coordinates, and similar sequences which act as a weak promoter in Tn2660 is shown in Fig. 5.

The minor transcripts from 3797 to 3800 are less easy to explain. The most appropriate rationale would be to conclude that the -35 consensus region is more complex and extends over 10 bases of a 12 base sequence as shown in Fig. 5 (30). The weaker promoter Pb, resulting in transcription from 3797 to 3800 would then be concluded to have arisen by a mutation of C to T in the proximal base of this -35 region, to result in a seven-base fit with the canonical sequence (4 minor, 3 major) and a 5 base fit (2 minor, 3 major) with the 9 canonical bases constituting a -10 (modified) Pribnow region (30). A comparison of the -35 and -10 sequences of this new promoter in Tn2661 and the corresponding sequences in Tn2660 is shown in Fig. 5.

The -10 sequence of Tn2660, GACAAT, identified at 3903 to3908 (13,14) to result in the initiation of transcription at 3915, is confirmed by our experiments. The -35 box, TTCAA of Tn2660 (coordinate 3880 to 3884) shows a three-base fit with the more conserved canonical -35 sequence.

From the size of the DNA probe protected by pBR322 mRNA shown in Fig. 3, it may be concluded that the pBR322 mRNA hybridizes with the entire segment of the HpaII fragment of Tn3 with which it is known to have homology. Thus, this promoter can be inferred to be located behond the EcoRI site of pBR322 in the region originating from pSC101, and is not present on Tn3, in accord with previous conclusions (14,15,27). However, in contrast to some of these previous findings (15,27) which have concluded that this promoter (termed P1, 27) has the same strength as the P3 promoter of Tn3, we see at best only a weak band corresponding to initiation from P3 in pBR322. Moreover, we find the relative intensity per unit weight of RNA of the band inferred from initiation at P1 in pBR322 to be fivefold greater

than that resulting from initiation from P3 in Tn2660. These relative intensities of these bands which may be concluded to reflect the relative amounts of pBR322 and Tn2660 mRNA moreover correspond to the relative ampicillin resistances shown by host strains carrying each of these elements. A similar correspondence is found in the relative intensities of the bands from protection by mRNAs from Tn2661 compared to Tn2660, of approximately 9 to 1, with the relative resistances conferred, which are 11 to 1. These correspondences between band intensities and expression suggest that the method of Berk and Sharp provides a reasonable measurement of the transcription level and therefore of promoter strength. However, the absence, or reduced intensity, of bands resulting from protection by mRNAs of Tn2661 or pBR322 initiated from P3 suggests that the intensities of bands derived by the Berk and Sharp method may well be dependent on other factors apart from relative promoter strengths. It might be suggested for example, that under conditions when the DNA probe, although present in excess, is not in gross excess, competition between transcripts would be seen. This would lead in some cases to a probe hybridized with a shorter transcript to undergo further hybridization with a longer transcript, leading to a preserved segment following S1 nuclease digestion corresponding to the longer transcript and in consequence, a relative loss in segments protected by the shorter transcript.

Comparison of the sequences in the -35 and -10 regions of P3, with those of Pa and Pb would nevertheless not appear to predict as great a difference in the relative strengths of the P3 and the Pa or Pb promoters as is found, and might suggest some cooperative activity of the overlapping Pa and Pb promoters.

Sequence of R6K and correspondence to TEM-1 enzymes

The similarities in base sequence and properties of Tn2660 (derived from R6K) and Tn3 (derived from R1) in the 3585/4023 region have been discussed earlier. R6K, and thus Tn2660, was the source of the original enzyme that was defined as TEM (3), and R6K with Tn2660 was later also used to define the TEM-1 subgroup (4). The enzyme from Tn3 also has the properties of a TEM-1 enzyme (4). From our base-sequence determinations of the other DdeI fragments (not shown), we conclude that the base at coordinate 4058 in Tn2660 is C, as was determined by Sutcliffe (11) for Tn3. Thus, the codon in Tn2660 at this position corresponds to the amino acid, Gln, as does that of Tn3, and supports the finding that the β-lactamase from Tn2660 (R6K) and Tn3 (R1) do not differ in pI and are both classed as TEM-1

enzymes (4). This strengthens the conclusion drawn by Sutcliffe (11) that the source of the β lactamase used for amino-acid sequence determinations by Ambler and Scott (31) was probably not R6K.

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